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CONJUGATION OF TRIAZIQUINONE TO IMMUNOGLOBULIN G BY A THIOLATION PROCEDURE CATALYZED BY 2-PYRIDINEALDOXIME METHIODIDE [1]

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Thiolation of immunoglobulin G (IgG) with DL-N-acetylhomocysteinethiolactone, catalyzed by 2-pyridinealdoxime methiodide, incorporated new sulfur groups into IgG. Triaziquinone was subsequently conjugated to the sulfur groups. Triaziquinone-IgG complex retained the alkylating activity of the drug and the immunological activity of the antibody. The conjugation procedure was inhibited by the thiol-blocking agent methyl methanethiolsulfonate.

INTRODUCTION

The immunologic response to certain diseases appears ineffective as a means of control and chemotherapy of disease often carries the hazard of systemic toxicity. These two problems could potentially be reduced by conjugating a chemotherapeutic compound to appropriate antibody. Hopefully, the antibody would carry the chemotherapeutic agent to the target antigen within the host. Therefore, antibodies would become more specifically cytotoxic while simultaneously diminishing the systemic toxicity associated with many chemotherapeutic agents.

This type of chemoimmunotherapy, conceived by Paul Ehrlich in 1906 (Ehrlich, 1906), has been evaluated with viral, bacterial, parasitic and neoplastic models. As reviewed by Rubens (1974), agents successfully conjugated and 'targeted' by antibodies have included various cytotoxic drugs,

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radioisotopes, boron, toxins and enzymes. Recently triaziquinone has been conjugated by a reduction procedure to antibodies directed to sarcoma tumor cells (Linford et al., 1974).

Potential problems exist in conjugating triaziquinone to immunoglobulin G (IgG) by a reduction process that relies on sulfur groups already present in IgG (Linford et al., 1974). The reduction procedure could weaken or completely degrade the IgG polypeptide into its component peptides if the process preferentially reduced the interchain rather than intrachain disulfide bonds. Interchain disulfide bonds are generally more susceptible to the effects of reducing agents than intrachain disulfide bonds (Konigsberg, 1972). Even though antibody specificity may return upon oxidation of reduced antibody fragments (White, 1972a), the procedure may decrease a portion of the immunological activity of the antibody. A loss of specific antibody activity would increase the significance of the cytotoxicity contributed by triaziquinone when bound to IgG of different specificities. The cytotoxicity of drug bound to non-specific IgG does not depend on a firm attachment of the complex to an antigen of a target cell (Linford and Froese, 1978).

A procedure is described in this report which conjugates triaziquinone to new sulfur groups on IgG (Fig. 1). The new sulfur groups are incorporated into the IgG molecule by a protein thiolation process involving DL-*N*-acetylhomocysteinethiolactone (White, 1972b). 2-Pyridinealdoxime methiodide catalyzes the reaction to favor the successful thiolation of the protein and to decrease the hydrolytic degradation of DL-*N*-acetylhomocysteinethiolactone (Klotz and Elfbaum, 1964). The triaziquinone-IgG complex retains the alkylating activity of the drug and the immunological activity of the antibody. The conjugation procedure is inhibited by the introduction of the thiol-blocking agent, methyl methanethiolsulfonate, immediately after the protein is thiolated by DL-*N*-acetylhomocysteinethiolactone.

MATERIALS AND METHODS

Virus production and purification

Influenza A/AA/6/60 H_2H_2 was obtained from Dr. Hunein F. Maassab, Department of Epidemiology, School of Public Health, The University of Michigan. It was originally isolated from a throat washing which was used to infect a chick kidney monolayer. The virus was subsequently passed 24 times in the allantoic cavity of chicken eggs.

A pool of virus was produced from the stock virus by propagating the virus in the allantoic cavity of fertile eggs. The stock virus possessed a 1024 hemagglutinating titer per 0.025 ml and had an EID_{50} (50% egg infectious dose) of 10^{-7} as measured by the Reed-Muench technique (Reed and Muench, 1938). A 10^{-7} dilution of stock virus was prepared in 0.3% nutrient broth, containing 100 U/ml of penicillin and 200 μg/ml of streptomycin.

A 0.1 ml volume of the diluted virus was inoculated into the allantoic cavity of viable 11-day-old Heisdorf-Nelson eggs (Singing Hills Poultry Farm, Manchester, MI). The allantoic fluid was collected after 3 days of incubation at 35° C. These virus pools had a 1024 to 2048 hemagglutinating titer per 0.025 ml and an EID_{50} of 10^{-7} as measured by the Reed-Muench technique.

The virus was purified by adsorption and elution with a 5% (by volume) suspension of chicken erythrocytes, followed by differential centrifugation at 5000 × *g* for 10 min and 75,000 × *g* for 1 h. The viral pellet was sonicated for 10 min at an output current of 1.0 A in a Raytheon sonic oscillator (Raytheon Manufacturing Company, Waltham, MA). The sonicated viral pellet was purified further by isopycnic centrifugation at 60,000 × *g* for 18 h in a 10—60% (w/v) continuous sucrose gradient.

Virus quantitation

Virus hemagglutinin was quantitated by the microhemagglutination assay (Sever, 1962). Infective virus was quantitated by the 50% egg infective dose assay (Reed and Muench, 1938).

Antibody production and purification

Male, New Zealand White rabbits (Johnson Rabbitry, Coldwater, MI), weighing approximately 5 lb, were used for production of antibody to purified influenza virus. Purified virus pooled from the isopycnic gradient that formed the peak between 1.1800 g/ml and 1.2000 g/ml, with a microhemagglutinating titer of 1024 per 0.025 ml, was injected into a lateral ear vein. A 2.0 ml primary injection was followed 1 week later by a 1.0 ml secondary injection. Subsequent injections of 1.0 ml were given every few months. Animals were bled weekly from a medial ear artery.

IgG was isolated from equilibrated serum by ion-exchange chromatography (Joustra and Lundren, 1969) with QAE Sephadex A-50 (Pharmacia Fine Chemicals Inc., Piscataway, NJ). Eluted IgG was concentrated in an Amicon model 52 stirred cell (Amicon Corporation, Lexington, MA) with an Amicon XM 100A anisotropic filter. The concentrated material was lyophilized and hermetically sealed in glass ampules.

Purity of the IgG was examined by slide immunoelectrophoresis (Williams and Chase, 1968). Purified IgG formed one line of precipitation of analogous position with either goat antiserum to rabbit whole serum (Microbiological Associates, Bethesda, MD) or goat antiserum to the heavy chain of rabbit IgG (Cappel Laboratories, Downingtown, PA).

Antibody quantitation

The presence of antibody to influenza virus was measured by the microhemagglutination inhibition assay (Sever, 1962). The amount of protein

contained in an IgG preparation was determined spectrophotometrically with a Beckman Acta Three spectrophotometer (Beckman Instrument Inc., Fullerton, CA) using a 280 nm and 260 nm nomograph (California Corporation for Biochemical Research, Los Angeles, CA).

Reduction procedure of conjugating triaziquinone to IgG

The reduction procedure (Linford et al., 1974) to conjugate triaziquinone (2,3,5-tris-ethylenimino-1,4-benzoquinone) to antibody was tried initially. The triaziquinone was obtained from Bayer Pharmaceuticals (Fahrenfabriken Bayer, Leverkusen, F.R.G.) and from the Drug Research and Development Division of the National Cancer Institute. Several alterations (from personal communications by Dr. J.H. Linford) were incorporated in the procedure. These alterations included: variation of the dithiothreitol (Calbiochem, San Diego, CA) concentration from 100 mM to 600 mM; using different lots of dithiothreitol; decreasing the incubation time with dithiothreitol from 90 to 15 min; more rapid removal of the excess dithiothreitol from the reaction mixture by chromatography on Bio Gel P-30 (Bio Rad Laboratories, Richmond, CA); and by adding a 2.0 M glucose concentration (Calbiochem, San Diego, CA) to the IgG before being exposed to the reducing agent.

Thiolation procedure of conjugating triaziquinone to IgG

A buffer of 0.1 M ammonium bicarbonate and 0.002 M ethylenediaminetetraacetic acid tetrasodium salt was prepared with double distilled water. The water was previously deoxygenated by boiling for 1 h and percolating with nitrogen gas for 1 h. This process removed 92% of the oxygen from the water as determined by the azide modification of the Winkler assay (American Public Health Association et al., 1975). A 200 mM 2-pyridinealdoxime methiodide (Aldrich Chemical Company Inc., Milwaukee, WI) and 0.125 mM IgG solution was prepared in the deoxygenated buffer, and enough DL-*N*-acetylhomocysteinethiolactone (Aldrich Chemical Company Inc., Milwaukee, WI) was added to this mixture to form a 250 mM concentration. The reaction mixture was incubated 4 h at 4° C with stirring. Nitrogen gas was continuously introduced into the reaction vessel. The pH was adjusted to 9.0 at 30 min intervals by a 5% trimethylamine solution (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH), which was also prepared in the deoxygenated buffer. After incubation, the mixture was dialyzed overnight against 3 changes of 300 ml of deoxygenated buffer. An equal volume of 4.3 mM triaziquinone was prepared in deoxygenated phosphate buffer and added to the reaction mixture under a nitrogen atmosphere. The reaction vessel was sealed and placed in a 37° C water bath for 4 h after which the mixture was dialyzed overnight against 3 changes of 300 ml of phosphate buffer.

Alkylation assay of drug activity

The alkylating activity of the triaziquinone was measured by the quaternization reaction (Linford, 1973). The only modification of the procedure was the use of an undiluted 1.0 ml sample of any preparation containing triaziquinone. 4-(*p*-nitrobenzyl)-pyridine was obtained from Aldrich Chemicals, Milwaukee, WI; 1,2-propanediol and triethylamine were obtained from Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH.

Blockage of sulfur groups

The thiol-blocking agent, methyl methanethiolsulfonate (Aldrich Chemical Company Inc., Milwaukee, WI), was used to analyze the mechanism by which the thiolation process conjugated triaziquinone to IgG (Smith et al., 1975).

RESULTS

Repeated efforts to conjugate triaziquinone to IgG by the reduction procedure were unsuccessful. The authors believe that the reducing agent, dithiothreitol, was denaturing the IgG molecule by the preferential reduction of the more susceptible interchain disulfide bonds (Konigsberg, 1972). If a random reoxidation of these interchain bonds occurred as the dithiothreitol was removed from the reaction mixture by dialysis, it could explain the observable precipitate that was formed and the unavailability of the sulfur groups in IgG to bind the sixth carbon of the triaziquinone molecule as hypothesized by Linford (1973).

Therefore, the thiolation procedure depicted in Fig. 1 was developed to conjugate triaziquinone to IgG. With the thiolation method, the quaternization assay for alkylating drugs detected the presence of 0.43 mM of active triaziquinone associated with IgG (Table 1). This represented an average of 7 active triaziquinone groups on each IgG molecule. If the IgG was not previously thiolated by 250 mM of DL-*N*-acetylhomocysteinethiolactone, no triaziquinone was found associated with the IgG. When 200 mM of 2-pyridinealdoxime methiodide was omitted from the thiolation procedure, 0.31 mM of drug was bound to IgG (Table 1). The absence of catalyst from the reaction produced a 25% decrease in the amount of drug conjugated to antibody.

Conjugation of triaziquinone to IgG by the thiolation procedure was prevented by the introduction of a thiol-blocking agent immediately after the protein was thiolated by DL-*N*-acetylhomocysteinethiolactone. Thiolated IgG was exposed to 1.62 mM of methyl methanethiolsulfonate for 30 min at 4°C. After the excess methyl methanethiolsulfonate was removed by overnight dialysis against deoxygenated buffer, the quaternization assay detected no subsequent binding of triaziquinone to IgG (Table 2). The

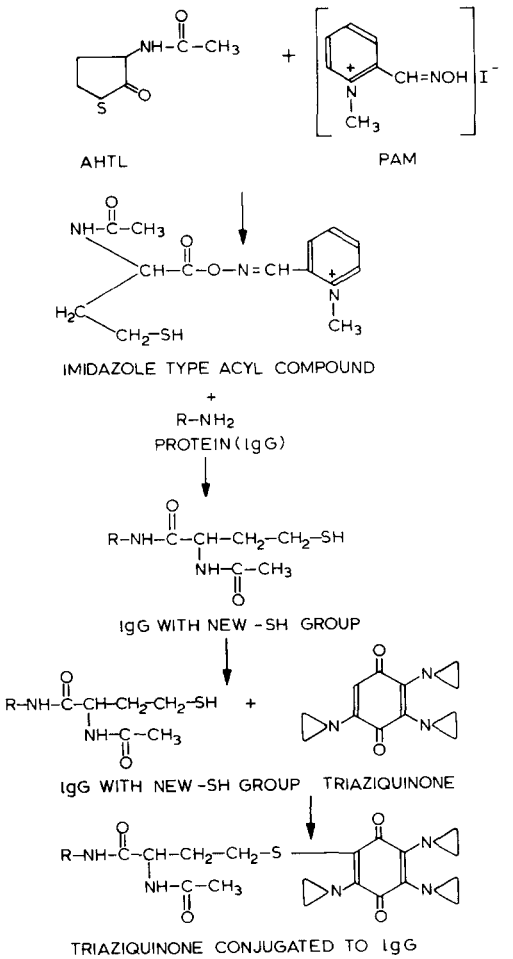

Fig. 1. Diagram of the hypothesized mechanism by which the thiolation procedure conjugates triaziquinone to IgG. The ring of the thiolating agent, DL-*N*-acetylhomocysteinethiolactone (AHTL), is opened by 2-pyridinealdoxime methiodide (PAM). The thiolating agent introduces a new sulfur on the IgG molecule which subsequently binds the triaziquinone.

lack of detectable triaziquinone on the IgG molecule was not caused by direct inactivation of the drug by the thiol-blocking agent. When triaziquinone was directly exposed to methyl methanethiolsulfonate, 0.43 mM of triaziquinone (100 μg/ml) was not measurably inactivated even after 1 h of exposure to 1.62 mM of methyl methanethiolsulfonate at 25° C. If IgG was exposed to the thiol-blocking agent before the antibody was thiolated, only a 20% decrease was observed in the subsequent amount of triaziquinone conjugated to IgG.

The effect of the thiolation procedure on the immunological activity of

TABLE 1

Conjugation of triaziquinone to protein by the thiolation procedure under various conditions

Type of experiment [a]	Protein type	Protein (mM)	AHTL (mM) [b]	PAM (mM) [c]	Triaziquinone (mM)	Triaziquinone bound (mM) [d]	Molecular ratio of bound triaziquinone/ protein
Conjugation of IgG	IgG	0.062	250	200	2.125	0.43	7
No AHTL bridge	IgG	0.062	0	200	2.125	0	0
No PAM catalyst	IgG	0.062	250	0	2.125	0.31	5

[a] Triaziquinone was conjugated to IgG by the thiolation procedure.
[b] DL-N-acetylhomocysteinethiolactone.
[c] 2-Pyridinealdoxime methiodide.
[d] The amount of triaziquinone bound to the protein was measured by the quaternization reaction.

TABLE 2

Effect of methyl methanethiolsulfonate on the thiolation of IgG and subsequent conjugation to triaziquinone

Time of exposure to MMTS	Bound triaziquinone (μg/ml) [d]	Per cent inhibition of conjugation
No exposure [a]	64	0
Before thiolation of IgG [b]	51	20
After thiolation of IgG [c]	0	100

[a] Triaziquinone was conjugated to IgG by the thiolation procedure.
[b] IgG was exposed to 1.62 mM of methyl methanethiolsulfonate (MMTS) for 30 min at 4°C before the IgG was thiolated by the thiolation procedure.
[c] IgG was exposed to 1.62 mM of methyl methanethiolsulfonate (MMTS) for 30 min at 4°C after the IgG was thiolated by the thiolation procedure.
[d] The amount of triaziquinone subsequently conjugated to the IgG by thiolation was determined by the quaternization reaction.

IgG was examined by the microhemagglutination inhibition assay. The anti-influenza IgG and triaziquinone-conjugated anti-influenza IgG had the same antibody titer of 64 per 0.025 ml as determined by the inhibition assay (Table 3). If the temperature of the thiolation reaction was raised to 25°C, the assay detected a decrease in antibody activity from 16 and 32 to a titer of 8 and 16 per 0.025 ml. The conjugation of drug to non-specific

TABLE 3

Effect of the conjugation process on the immunological activity of antibody

Type of antibody [a]	Antibody conjugated to triaziquinone	Thiolation temperature [b] (°C)	IgG (mg/ml) [c]	Antibody titer per 0.025 ml [d]
Influenza	No	4	8.0	64, 64
Influenza	Yes	4	8.0	64, 64
Non-specific	No	25	8.4	0, 0
Non-specific	Yes	25	8.4	0, 0
Influenza	No	25	8.4	16, 32
Influenza	Yes	25	8.4	8, 16

[a] IgG was purified by ion-exchange chromatography with QAE Sephadex A-50 from normal rabbit serum or from rabbits immunized with influenza virus.
[b] Triaziquinone was conjugated to IgG by the thiolation procedure. One batch of IgG was thiolated at 4°C and another batch of IgG was thiolated at 25°C.
[c] IgG concentration was determined spectrophotometrically by using a 280 nm and 260 nm nomograph.
[d] Immunological activity of antibody was measured in duplicate by microhemagglutination inhibition.

IgG did not induce the IgG to adsorb influenza virus. Both the non-conjugated non-specific IgG and non-specific IgG conjugated to triaziquinone had antibody titers of 0.

DISCUSSION

The significance of this exciting area of chemoimmunotherapy research is that various types of cytotoxic agents could potentially be conjugated to antibodies. Even the multitude of cytotoxic drugs that have never reached the stage of clinical trial, or those removed from the pharmaceutical market because of unforeseen systemic toxicity problems, may be satisfactory agents for chemoimmunotherapy. Relying on the immunological specificity of the antibody, the cytotoxic agent would be preferentially delivered to the site of the target antigen in the patient. The target could either be a viral, bacterial, parasitic, or neoplastic antigen.

During the preparation of cytotoxic agents conjugated to antibody carriers, one must be careful to preserve the cytotoxic quality of the cytotoxic agent as well as the immunological activity of the antibody. Because a reduction procedure of conjugating IgG and triaziquinone (Linford et al., 1974) may lead to a random reoxidation and subsequent denaturation of the peptide components of IgG, a new thiolation procedure was developed, as described here, which relies on exogenous instead of the endogenous sulfur groups in IgG.

The thiolation procedure apparently conjugated 0.43 mM of active triaziquinone to IgG as determined by the quaternization assay for alkylating drugs (Fig. 1). Unless the IgG was previously thiolated by DL-*N*-acetylhomocysteinethiolactone, the quaternization assay detected no active drug bound to the IgG (Table 1). This implies that the conjugation of triaziquinone to IgG was not a result of adsorption of the drug to the protein.

The hypothesized mechanism of the binding of drug by the new sulfur groups was confirmed by experiments involving the thiol-blocking agent (Smith et al., 1975). When thiolated IgG was exposed to 1.62 mM of methyl methanethiolsulfonate, no triaziquinone was subsequently detected on the IgG molecule by the quaternization reaction (Table 2). Presumably the sulfur groups incorporated into the IgG molecule by DL-*N*-acetylhomocysteinethiolactone were not available to link the triaziquinone. The absence of detectable drug bound to the antibody in these experiments was not explainable by methyl methanethiolsulfonate directly inactivating the triaziquinone, since a 1.62 mM concentration of the blocking agent did not completely inactivate the alkylating activity of 0.43 mM (100 μg/ml) of the drug. When IgG was exposed to the thiol-blocking agent before the initiation of thiolation procedure, there was a 20% decrease in the amount of drug subsequently bound to IgG (Table 2). That 20% decrease probably represents a steric hindrance of the thiolation procedure by the thiol-blocking agent binding to accessible sulfur groups originally present in the antibody.

Whereas the Linford process relies on the reduction of endogenous sulfur groups in the IgG structure (Linford et al., 1974), the conjugation of triaziquinone to IgG, by using a thiol bridge, relies on previously published thiolation reactions to incorporate new sulfur groups into the IgG molecule. The other proteins thiolated by DL-*N*-acetylhomocysteinethiolactone have included glycine (Benesch and Benesch, 1956), bovine serum albumin (Singer et al., 1960), ovalbumin (Singer et al., 1960), alpha chymotrypsinogen (Abadi and Wilcox, 1960), ribonuclease (Singer et al., 1960; White and Sandoval, 1962), insulin (Virupaksha and Tarver, 1964), and gelatin (Klotz and Elfbaum, 1964). Thiolated antibodies have been used as specific immunoadsorbents by subsequently cross-linking the new sulfur groups into disulfide bridges (Stephen et al., 1966; Chidlow et al., 1967; Wood et al., 1968). An average of 8—9 sulfur groups were incorporated on each IgG molecule in the antibody immunoadsorbents, although the range could be extended from 5 to 20 sulfur groups by varying the reaction conditions. The ability of 7 triaziquinone molecules to bind to 7 new sulfur groups, introduced by the thiolation procedure, is shown by our results in Table 1. The results, from either the reduction or thiolation process, is that the sulfur groups apparently provide a site for subsequent binding of the sixth carbon of the triaziquinone compound as hypothesized by Linford (1973).

The conditions of the present thiolation procedure did not favor subsequent disulfide bond formation by the new sulfur groups. The boiling and percolation of the aqueous solutions with nitrogen gas, as well as the nitrogen atmosphere in the reaction vessel, inhibited the oxidation of the new sulfur groups (Virupaksha and Tarver, 1964; Stephen et al., 1966; White, 1972a, b). Furthermore, ethylenediaminetetraacetic acid was present to chelate metals that might catalyze the oxidation of sulfur (Konigsberg, 1972). Disulfide bond formation by the presence of high protein concentration was avoided by using 0.062 mM protein concentrations (Stephen et al., 1966; White, 1972a). The reaction conditions also avoided the problem of intermolecular and intramolecular bond formation by the new sulfur groups with the sulfur groups already present on IgG (White and Sandoval, 1962).

The ammonium bicarbonate buffer was the first and only buffer utilized in the study since it was the buffer chosen by the initial investigators who thiolated proteins with acetylhomocysteinethiolactone (White and Sandoval, 1962; White, 1972b). Another buffer was never sought since triaziquinone was succesfully conjugated to IgG while using this buffer. Any nucleophilic character of this buffer would probably be overshadowed in the thiolation procedure by the pyridinealdoxime methiodide, the imidazole catalyst, which itself is known as a nucleophile (Klotz and Elfbaum, 1964).

The reaction conditions favored the successful thiolation of IgG by the aminolysis of DL-*N*-acetylhomocysteinethiolactone in comparison to the destructive hydrolysis of the thiolating agent. Since imidazole type com-

pounds can split thioester linkages, 2-pyridinealdoxime methiodide was added as a catalyst to form an active acylimidazole intermediate by opening the ring of DL-*N*-acetylhomocysteinethiolactone (Klotz and Elfbaum, 1964). A 25% increase in the amount of triaziquinone bound to IgG was observed by the addition of the catalyst to the reaction (Table 1). Imidazole catalysts are made more effective by increasing the pH of the reaction to 9 (Klotz and Elfbaum, 1964). The 5% trimethylamine buffer has great buffering capacity at the alkaline pH (White and Sandoval, 1962). High pH also hinders the hydrolytic degradation of thiolating agents (Benesch and Benesch, 1956). The 4°C temperature of the reaction favored aminolysis since the temperature coefficient of the rate of aminolysis is small (Benesch and Benesch, 1956).

Thiolated antibodies retain their immunological activity (Stephen et al., 1966; Chidlow et al., 1967; Wood et al., 1968). The binding of triaziquinone to the new sulfur groups apparently did not affect the antibody's reactivity to influenza virus. The non-conjugated IgG and IgG conjugated to triaziquinone had the same titer of 64 per 0.025 ml, as determined by the microhemagglutination inhibition assay (Table 3). DL-*N*-acetylhomocysteinethiolactone can bind the epsilon or alpha nitrogen of proteins (White, 1972a). Since thiolated IgG prepared at 4°C retained the immunological activity that is associated near the alpha nitrogen position, the thiolating agent was probably bound to the epsilon nitrogen of IgG. Even though the alpha nitrogen may be somewhat distant from the actual antibody combining site, the conjugation of a large prosthetic group to this area of the antibody structure, consisting of a thiol bridge and triaziquinone molecule, could still be near enough to the actual combining site of the IgG to have deleteriously affected its affinity and avidity. Indeed, the partial loss of immunological activity of the conjugation products prepared at 25°C may be possibly explained, in part, by thiol and triaziquinone groups being able to bind to the less reactive alpha nitrogens of IgG at this elevated reaction temperature (Table 3). The thiolation procedure's predilection for the epsilon position may have been expected since the epsilon amino groups are generally more basic or nucleophilic (Lawton, R.G., personal communication, 1977) as well as usually occurring more often in proteins than alpha amino groups. The conjugation of triaziquinone to thiolated IgG did not cause non-specific IgG to adsorb influenza virus. Neither the non-specific IgG, nor non-specific IgG conjugated to triaziquinone, bound influenza virus, as determined by the microhemagglutination assay (Table 3). Thiolated antibodies used as specific immunoadsorbents have not adsorbed proteins non-specifically (Stephen et al., 1966; Chidlow et al., 1967; Wood et al., 1968).

Our immediate interest is to analyze the activity of the conjugation product on tumor cells in vitro by conjugation triaziquinone to anti-tumor immunoglobulin G. If any enhancement in specific cytotoxicity is exhibited by the conjugation product in comparison to treatment of the target cells

with triaziquinone and anti-tumor antibody not conjugated together, anti-tumor antibody alone or triaziquinone alone, the thiolation process as well as the conjugation product will be characterized further. This will include the analysis of the optimum reaction conditions for the thiolation procedure to retain the alkylating and immunological activity of the conjugation product, the number and location of new thiol groups introduced in the IgG molecule by using Ellman's reagent and amino acid analysis, and the number and location of active as well as inactive triaziquinone groups subsequently conjugated to these thiol groups.

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